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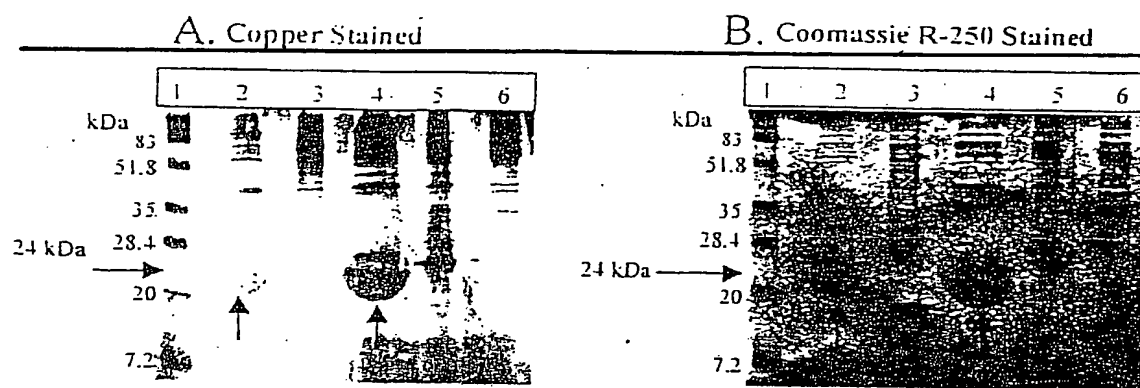
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# Figure 1: Biopolymer-Proinsulin Fusion Protein Expression



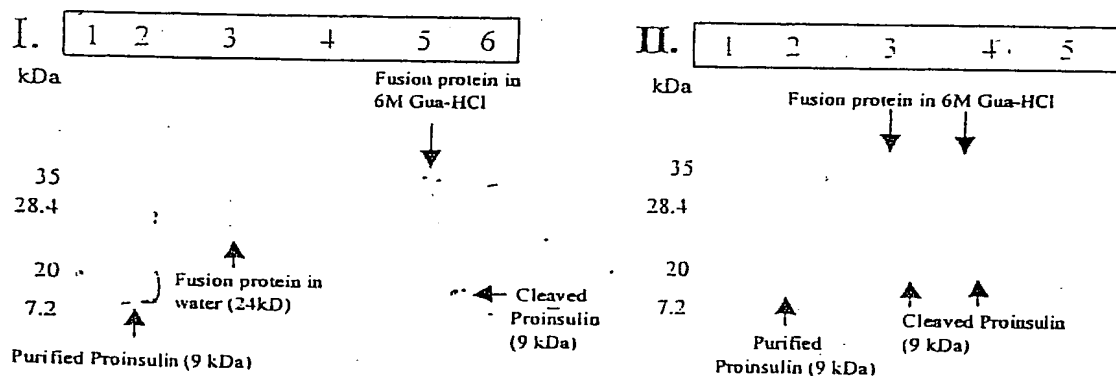
PAGE: 15% Glycine large gel

**A. Copper Stained:** Gel rinsed in water for 10 min, stained with 0.3M  $\text{CuCl}_2$  for 5min, and rinsed in water for 3min.

**B. Coomassie R-250 Stained:** The same gel was first rinsed for 20min in water and then stained for 1hr, and destained overnight.

**A. and B. Lanes. 1, Prestained Marker (BioRad); 2, Sonic extract of pSBL-OC-XaPris; 3, reverse orientation of fusion protein of pSBL-OC-XaPris; 4, Sonic extract of pLD-OC-XaPris; 5, inverse orientation of pLD-OC-XaPris; 6, Sonic extract of *E. coli* strain XL-1 Blue containing no plasmid.**

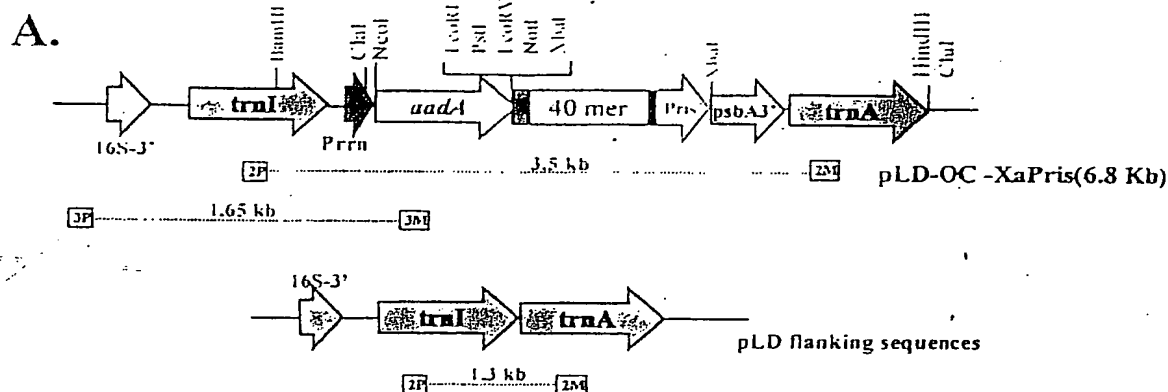
## C. Western Blot of Biopolymer-Proinsulin Fusion Protein



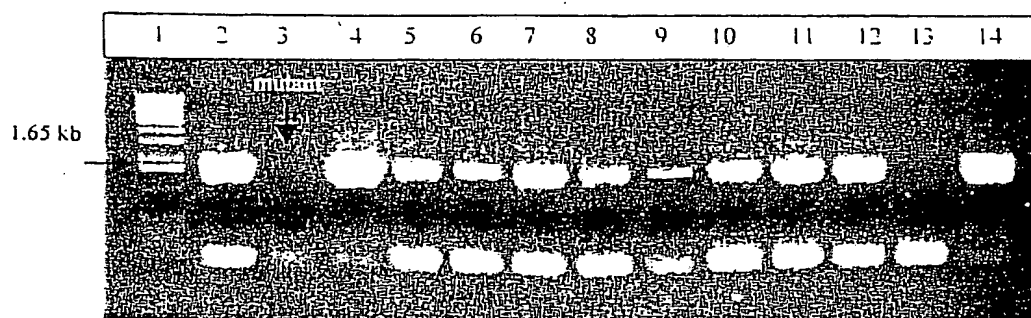
**I. Lanes. 1, BioRad Prestained Marker; 2, 3ug of Purified Human Proinsulin; 3, 5ug of pSBL-OC-XaPris (sonication and purification of biopolymer twice); 4, Negative control, XL-1 Blue *E. coli*; 5, Sonic extract pSBL expressing cells (6M Guanidine Hydrochloride Phosphate Buffer, pH 7.0); 6, Sonic extract of XL-1 Blue *E. coli* with no pSBL.**

**II. Lanes. 1, BioRad Prestained Marker; 2, 5ug of Purified Human Proinsulin; 3, Sonic extract of pSBL-OC-XaPris expressing cells (6M Guanidine Hydrochloride Phosphate Buffer, pH 7.0); 4, Sonic extract of pLD-OC-XaPris expressing cells (Gua-HCl); 5, Sonic extract of XL-1 Blue *E. coli* with no plasmid.**

## Figure 2: Confirmation of Chloroplast Integration by PCR of Polymer-Proinsulin Fusion Gene

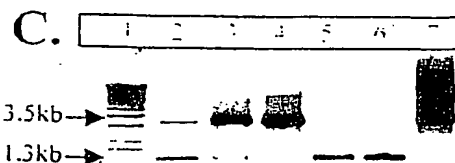


### B. Confirmation of *aadA* integration into the chloroplast genome - Primers: 3P/3M

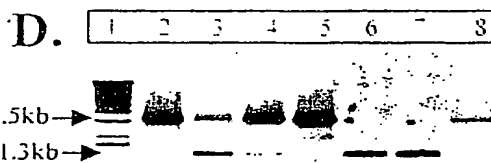


A. Lanes, 1, 1 kb marker; 2, clone L19b (L=pLD-OC-XaPris) vector0; 3, clone L9 (mutant); 4, L1; 5, L8d; 6, L10a; 7, S30b (S=pSBL-OC-XaPris vector); 8, S20a; 9, S60; 10, S7a; 11, S28; 12, S41b; 13, Petit havana (not transgenic); 14, Positive control (BADH gene present in chloroplasts from transgenic plants already confirmed)

### Confirmation of integration of *aadA* and biopolymer-proinsulin fusion genes into the chloroplast genome - Primers: 2P/2M

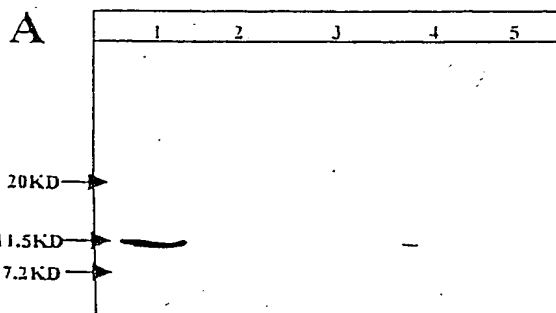


PCR of pLD clones: Lanes, 1, 1kb marker; 2, L17a; 3, L19b; 4, L8d; 5, L9; 6, Petit havana (not transgenic); 7, pLD vector as positive control



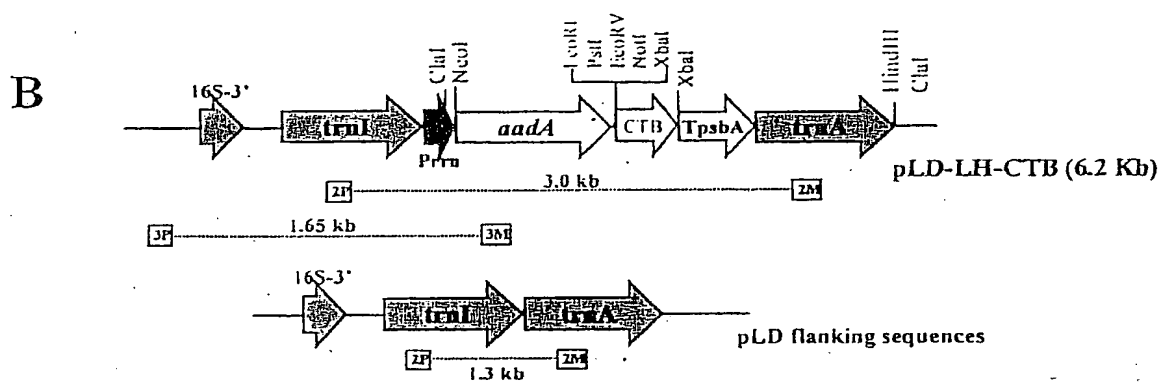
PCR of pSBL clones: Lanes, 1, 1kb marker; 2, S17a; 3, S30b; 4, S7a; 5, S41b; 6, L9(mutant); 7, Petit havana (not transgenic); 8, pSBL vector as positive control

### Figure 3 : CTB Gene Expression and Chloroplast Integration

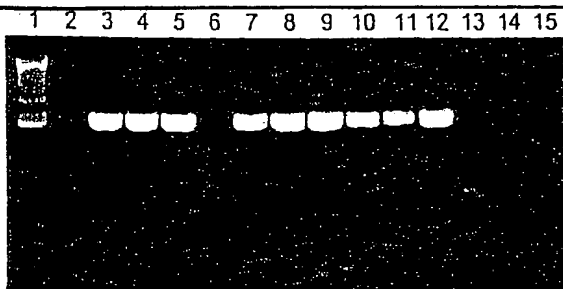


#### Western Blot analysis of CTB expression in *E. coli* (15% PAGE):

Lane 1: Purified bacterial CTB (0.5 µg); 2 & 4: Transformed *E. coli* culture-24 h and 48 h resp.; 3 & 5: Untransformed *E. coli* culture- 24 h and 48 h resp.



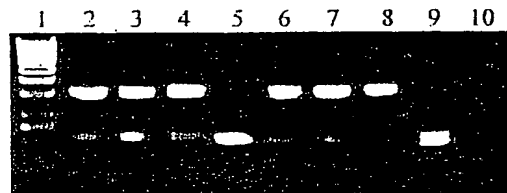
#### C. PCR confirmation of *aadA* gene integration into chloroplast genome -3P/3M primers



#### PCR of clones of 1st. round of selection :

Lane 1: 1 Kb marker; 2 - 12 : Plant total DNA from spec. clones 1-11 (Note: Lanes 2 & 6 are mutants); 13: Untransformed plant; 14: pLD-LH-CTB vector; 15: No DNA template

#### D. PCR confirmation of integration of *aadA* and CTB gene into chloroplast genome - 2P/2M primers



#### PCR of clones of 2nd. round of selection :

Lane 1: 1 Kb marker; 2 - 7 : Plant total DNA from spec. clones 1- 6 (Note: Lane 5 is a mutant); 8: pLD-LH-CTB vector; 9: Untransformed plant; 10 : No DNA template.

• Expression of bacterial operon in transgenic chloroplasts.

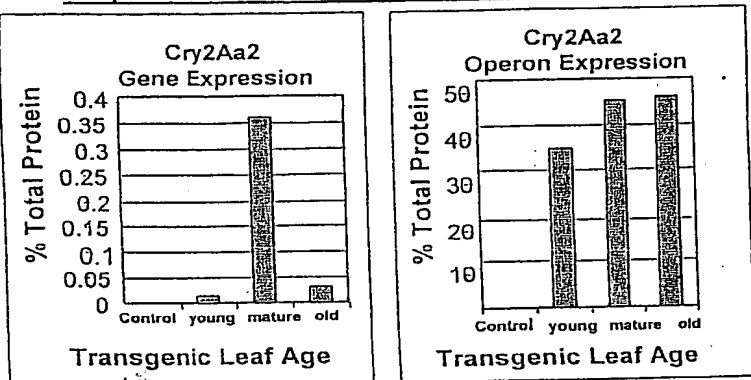


Figure 1: Cry2A protein concentration determined by ELISA in transgenic leaves. Note 100-fold increase in protein accumulation in the presence of the putative chaperonin, ORF2.

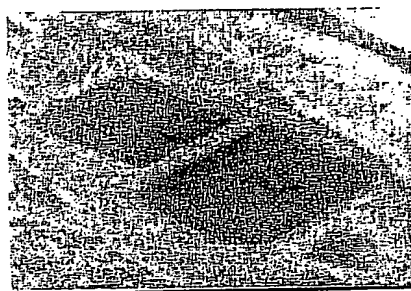


Figure 2: Immunogold labeled electron microscopy of mature transgenic leaf. Cry2Aa2 crystals in a transgenic chloroplast expressing the cry2A operon.

• Expression of a small (22aa) peptide in transgenic chloroplasts.

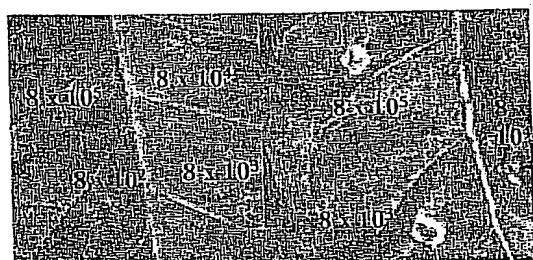


Figure 3: Leaves were infected with 10  $\mu$ l of  $8 \times 10^5$ ,  $8 \times 10^4$ ,  $8 \times 10^3$  and  $8 \times 10^2$  cells of *P. syringae*. Photos were taken 5 days after inoculation. 1-2  $\mu$ g of antimicrobial peptide (AMP) is required to kill 1000 bacterial cells. Local concentration at the site of infection is estimated to be 200-800  $\mu$ g AMP.

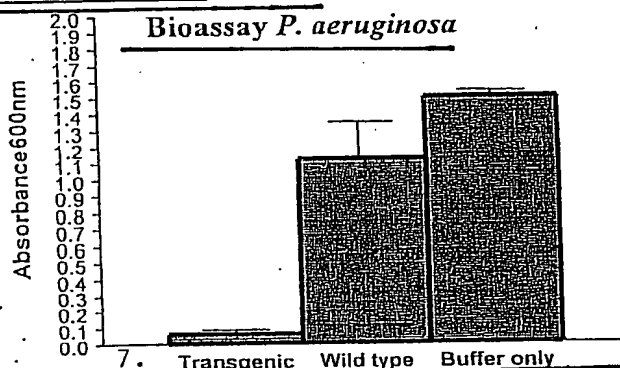


Figure 4: Total plant protein was mixed with 5  $\mu$ l of mid-log phase bacteria from overnight culture, incubated for 2 hours at 25°C at 125rpm and grown in LB broth overnight. Based on minimum inhibitory concentration of 1-2  $\mu$ g AMP/1000 bacterial cells, the expression level was calculated to be 21.5-43% of the total soluble protein.

• Expression of Oligomeric form (disulfide bonded) CTB in transgenic chloroplasts.

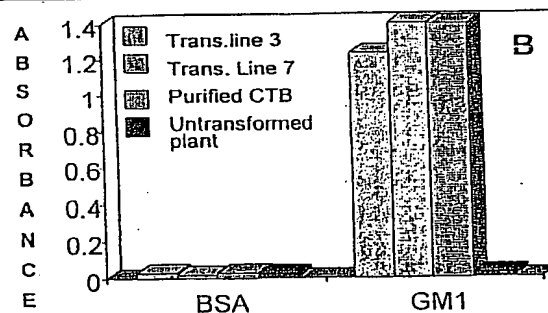
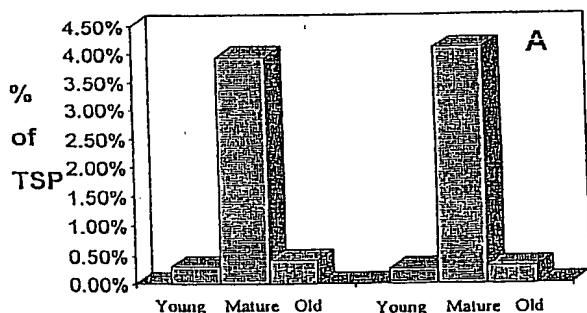


Figure 5: A) CTB ELISA quantification is shown as a percentage of the total soluble plant protein. Total soluble plant protein from young, mature and old leaves of transgenic lines 3 and 7 was quantified. B) CTB-GM1 Ganglioside binding ELISA assays: Plates coated first with GM1 gangliosides and BSA were plated with total soluble plant protein from lines 3 and 7, untransformed plant total soluble protein and purified bacterial CTB. The absorbance of the GM1 ganglioside-CTB antibody complex was measured.

# • Expression of CTB oligomers.

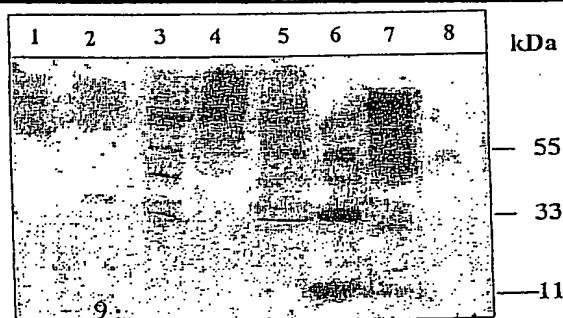


Figure 6: 12% reducing PAGE. Chemiluminescent detection with rabbit anti-cholera serum (1<sup>st</sup>) and AP labeled mouse anti-rabbit IgG (2<sup>nd</sup>) antibodies. Untransformed, boiled (1) and unboiled (2); Transformed, boiled (3&5) and unboiled (4); Purified CTB boiled (6) and unboiled (7); Marker (8).

# • Expression & assembly of disulfide bonded Guy's 13 monoclonal antibody.

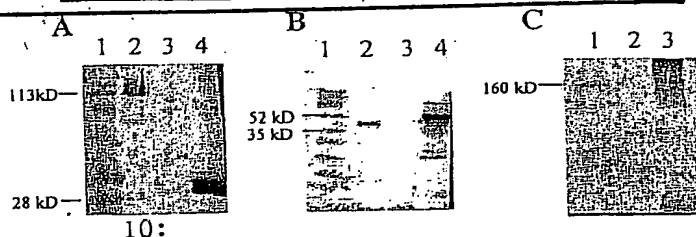


Figure 7: A, B) reducing gels. 1: markers, 2: Transgenic extract showing expression of light (A) and heavy chain (B) in chloroplasts. 3: Untransformed, 4: Human IgA. C) non-reducing gel. 1: Transgenic extract showing assembly, 2: Untransformed, 3: Human IgA. Blots A & C were detected with AP conjugated goat anti-human kappa antibody. Blot B was detected with AP conjugated goat anti-human IgA antibody.

# • Marker-free chloroplast transgenic plants.

Selectable marker	Plate No.	Total no. of leaf discs	No of responding leaf discs	Total no of shoots/ plate
BADH	1	3	3	43
	2	6	4	23
	3	11	9	33
	4	7	6	19
	5	6	4	16
	6	9	7	18
Spectinomycin	1	5	0	0
	2	5	0	0
	3	5	3	3
	4	5	2	2
	5	5	0	0
	6	5	1	1
	7	5	1	2
	8	5	1	2
	9	5	0	0
	10	5	0	0
Control		5	0	0

Table F: Comparison of Spectinomycin and Betaine aldehyde as the selectable marker for the first round of selection.

# • Codon composition and expression levels.

Open reading Frame	% TSP	% A+T	% psbA	% cp tRNA
Plastid miniprinsulin	?	66	100	62
CTB	4.1	66	47	34
Cry2A operon	47	65	37	37
Plastid proinsulin	?	64	100	49
Antimicrobial peptide	21	63	35	35
Guy's light chain	<1%	49	31	44
Optimized biopolymer	?	47	100	40
Guy's heavy chain	<1%	40	25	44
Human proinsulin	?	38	26	44

Table 2 (Left): Black indicates genes with unmodified native codon composition and their expression levels observed in transgenic chloroplasts, ranked by AT% in ascending order. Red indicates genes to be investigated. Kusnadi et al. (1997) suggest that a minimum of 1% TSP is adequate for commercial feasibility. See section d) for details of A1 content, %psbA optimal codons and % of codons that match the cp tRNA pool. TSP: % total soluble protein

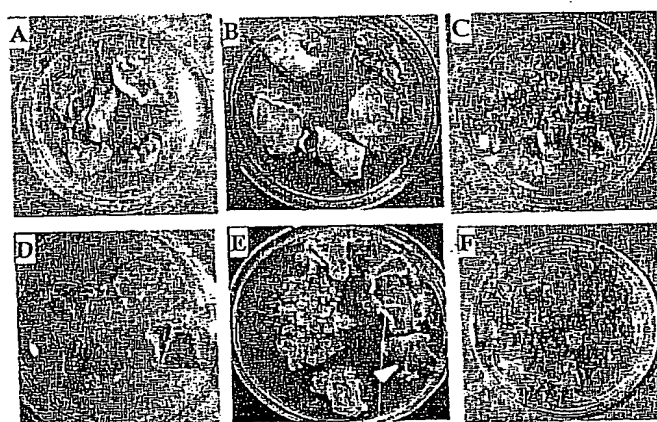


Figure 8: Comparison of betaine aldehyde and spectinomycin selection. A. *N. tabacum* Petit Havana control in RMOP medium containing spectinomycin after 45 days. B. Bombarded leaf discs selected on spectinomycin in RMOP medium after 45 days. C. Spectinomycin resistant clones cultured again (second round) to obtain homoplasmy. D. Petit Havana control in RMOP medium containing Betaine Aldehyde after 12 days of culture. E. Bombarded leaf discs selected on Betaine Aldehyde in RMOP medium after 12 days of culture; arrow indicates unbombarded leaf disc as control. Note that 23 shoots are formed on a disc selected on betaine aldehyde against 1-2 shoots per disc on spectinomycin. F. Betaine aldehyde resistant clones cultured again (second round) to obtain homoplasmy.

## Biopolymer-Proinsulin Fusion Protein Expression

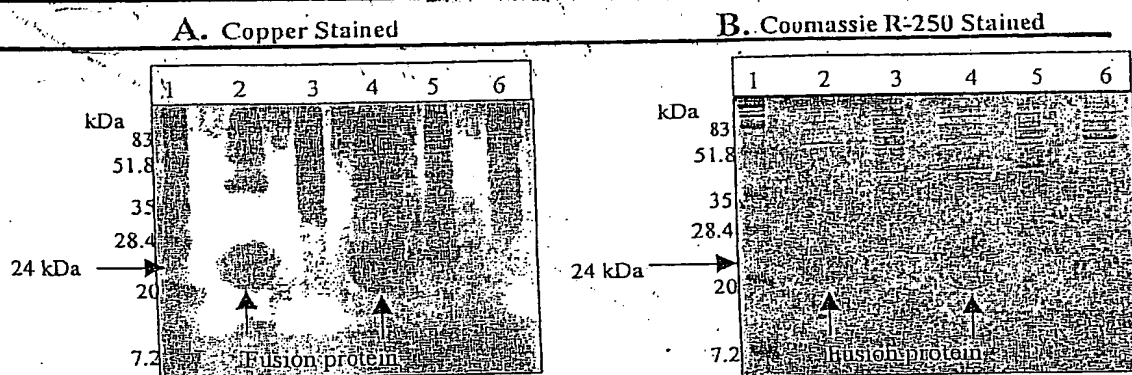


Fig 9, A and B Lanes: 1, Prestained Marker (BioRad) ; 2, Sonic extracts of pSBL-OC-XaPris ; 3, reverse orientation of insert in pSBL-OC- XaPris; 4, pLD-OC-XaPris; 5, reverse orientation of pLD-OC- XaPris; 6, *E. coli* XL-1 Blue cells with no plasmid .

## Western Blots of Biopolymer-Proinsulin Fusion Protein After Single Step purification

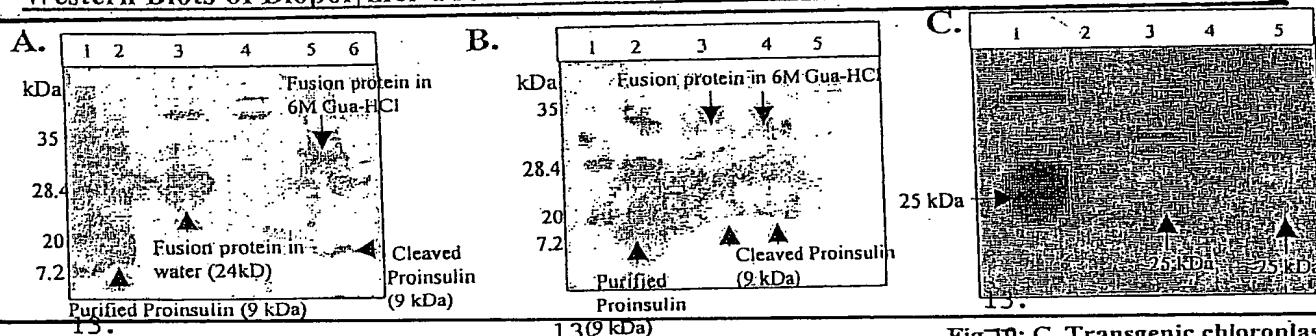


Fig 10: A. *E. coli* expression and cleavage Lanes: 1, BioRad Prestained Marker; 2, 3ug of Purified Human Proinsulin; 3, 5ug of pSBL-OC-XaPris; 4, Negative control, reverse orientation; 5, pSBL expressing cells (6M Guanidine Hydrochloride Phosphate Buffer, pH7.0); 6, XL-1 Blue *E. coli* with no pSBL.

Fig 10: B. *E. coli* expression and cleavage Lanes: 1, BioRad Prestained Marker; 2, 5ug of Purified Human Proinsulin; 3, pSBL OC-XaPris (6M Guanidine Hydrochloride Phosphate Buffer, pH7.0); 4, pLD- OC-XaPris; 5, XL-1 Blue *E. coli* with no plasmid.

Fig 10: C. Transgenic chloroplast expression Lanes: 1, Purified *E. coli* protein from pLD-OC-XaPris expression; 2, negative control (Petit Havana); 3-5, Chloroplast transgenic lines. Note dimer, tetramer and hexamer aggregates of polymer-insulin fusion protein

## Confirmation of chloroplast integration and homoplasmy/heteroplasmy by Southern Blot Analysis

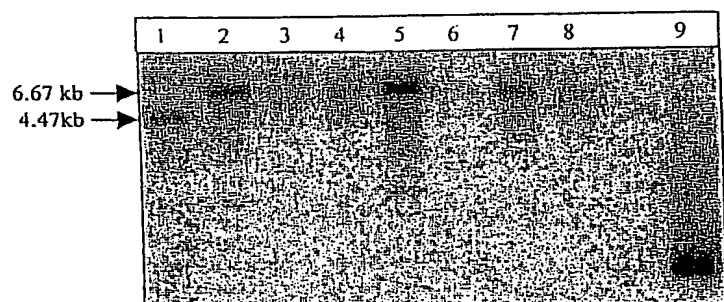


Fig11. Biopolymer-proinsulin fusion gene integration into the chloroplast genome confirmed by Southern blot analysis. Lanes: 1, Petit Havana (negative control); 2-5, pLD-OC-XaPris clones T<sub>0</sub>; 6-8, pSBL-OC-XaPris clones T<sub>0</sub>; 9, probe(positive control). Homoplasmy is seen in most transgenic lines while a few transgenic lines show heteroplasmy.

• Expression of bacterial operon in transgenic chloroplasts.

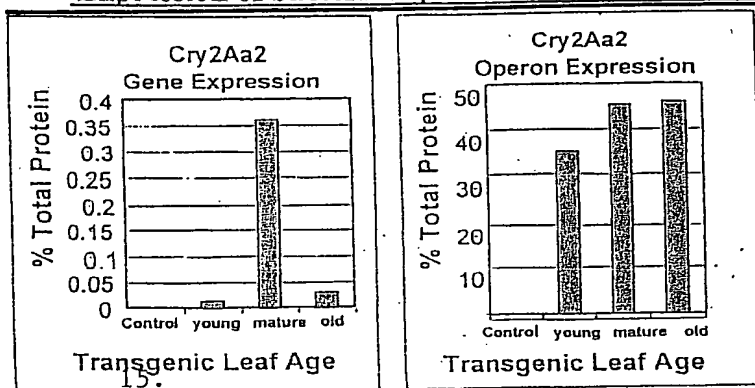


Figure 1: Cry2A protein concentration determined by ELISA in transgenic leaves. Note 100-fold increase in protein accumulation in the presence of the putative chaperonin, ORF2.

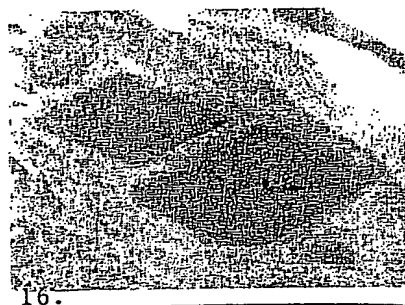


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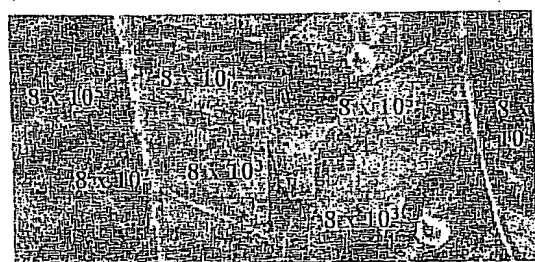


Figure 3: Leaves were infected with  $10 \mu\text{l}$  of  $8 \times 10^5$ ,  $8 \times 10^4$ ,  $8 \times 10^3$  and  $8 \times 10^2$  cells of *P. syringae*. Photos were taken 5 days after inoculation.  $1-2 \mu\text{g}$  of antimicrobial peptide (AMP) is required to kill 1000 bacterial cells. Local concentration at the site of infection is estimated to be 200-800  $\mu\text{g}$  AMP.

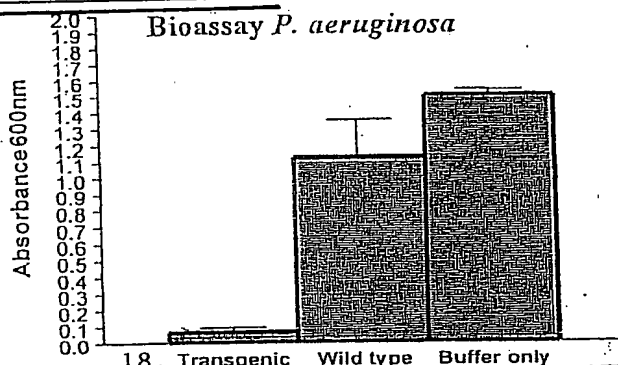


Figure 4: Total plant protein was mixed with  $5 \mu\text{l}$  of mid-log phase bacteria from overnight culture, incubated for 2 hours at  $25^\circ\text{C}$  at 125rpm and grown in LB broth overnight. Based on minimum inhibitory concentration of  $1-2 \mu\text{g}$  AMP/1000 bacterial cells, the expression level was calculated to be 21.5-43% of the total soluble protein.

• Expression of Oligomeric form (disulfide bonded) CTB in transgenic chloroplasts.

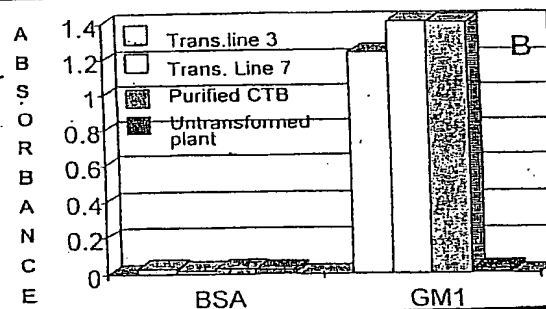
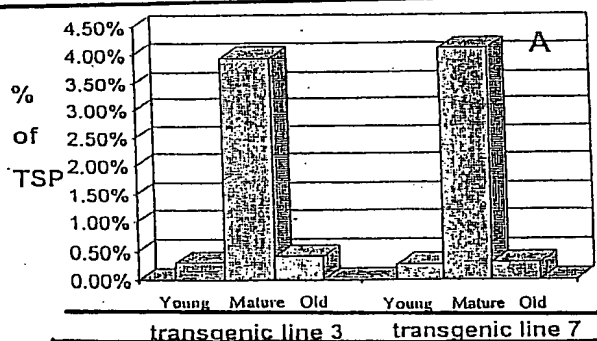


Figure 5: A) CTB ELISA quantification is shown as a percentage of the total soluble plant protein. Total soluble plant protein from young, mature and old leaves of transgenic lines 3 and 7 was quantified. B) CTB-GM1 Ganglioside binding ELISA assays: Plates coated first with GM1 gangliosides and BSA were plated with total soluble plant protein from lines 3 and 7, untransformed plant total soluble protein and purified bacterial CTB. The absorbance of the GM1 ganglioside-CTB antibody complex was measured.



### • Expression of CTB oligomers.

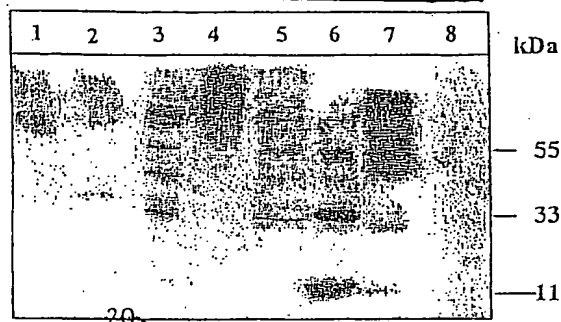


Figure 6: 12% reducing PAGE. Chemiluminescent detection with rabbit anti-cholera serum (1<sup>st</sup>) and AP labeled mouse anti-rabbit IgG (2<sup>nd</sup>) antibodies. Untransformed, boiled (1) and unboiled (2); Transformed, boiled (3&5) and unboiled (4); Purified CTB boiled (6) and unboiled (7); Marker (8).

### • Expression & assembly of disulfide bonded Guy's 13 monoclonal antibody.

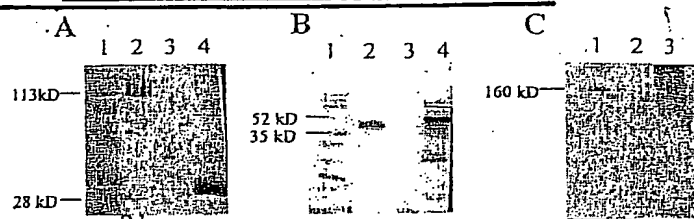


Figure 7: A, B) reducing gels. 1: markers. 2: Transgenic extract showing expression of light (A) and heavy chain (B) in chloroplasts. 3: Untransformed, 4: Human IgA. C) non-reducing gel. 1: Transgenic extract showing assembly, 2: Untransformed, 3: Human IgA. Blots A & C were detected with AP conjugated goat anti-human kappa antibody. Blot B was detected with AP conjugated goat anti-human IgA antibody.

### • HSA Nuclear transformation of potato plants.

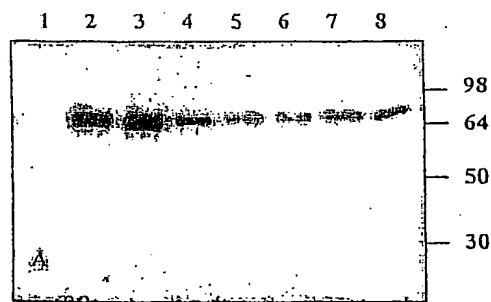


Figure 8: Western Blot of transgenic potato tubers. cv. Désirée. 30 µg of tuber protein was loaded per lane and probed with anti-HSA antibody. 1: wild type; 2: 40 ng of pure HSA; 3-8: different transgenic lines, showing different levels of expression.

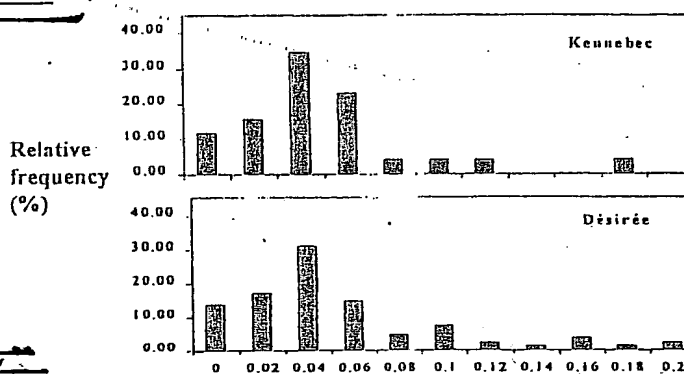


Figure 9: Frequency histogram including percentage Kennebec and Désirée transgenic plants expressing different HSA levels. Results are shown as the percentages of transgenic plants (vertical axis) that express a specific level of HSA of the total soluble protein (horizontal axis).

### • Expression of HSA by chloroplast vectors in *E. coli*.

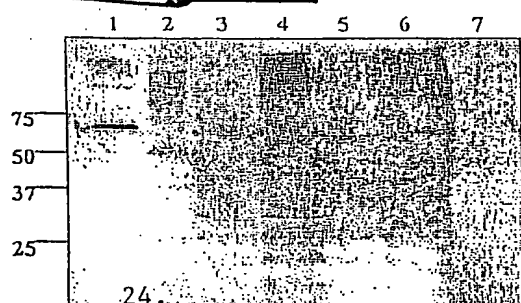


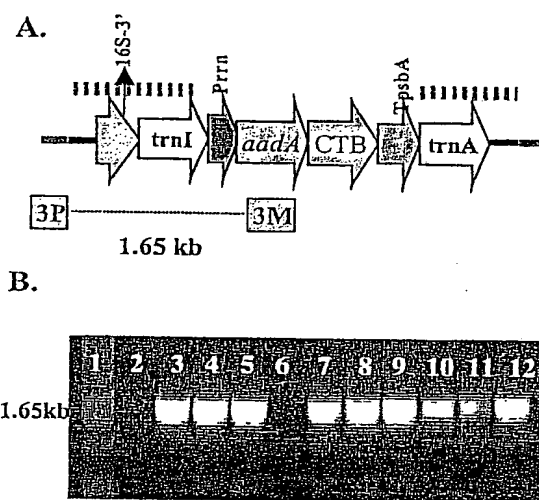
Figure 10: Western Blot of *E. coli* protein extracts. 1: 50 ng pure HSA; 2: molecular weight marker; 3: pLD-HSA (control without RBS); 4: pLD-5'UTR-HSA; 5: pLD-RBS-HSA; 6: pLD-ORF1+2-HSA; 7: *E. coli* without pLD vector.

### • Codon composition and expression levels.

Open reading Frame	% TSP	% A+T	% psbA	% cp tRNA
CTB	4	66	47	34
Cry2A operon	47	65	37	37
Antimicrobial peptide	21-43	63	35	35
HSA	?	57	57	47
Interferon alpha	?	54	31	40
RUBISCOssTP	?	50	32	42
Guy's light chain	<1%	49	31	44
IGF-I	?	41	20	30
Guy's heavy chain	<1%	40	25	44

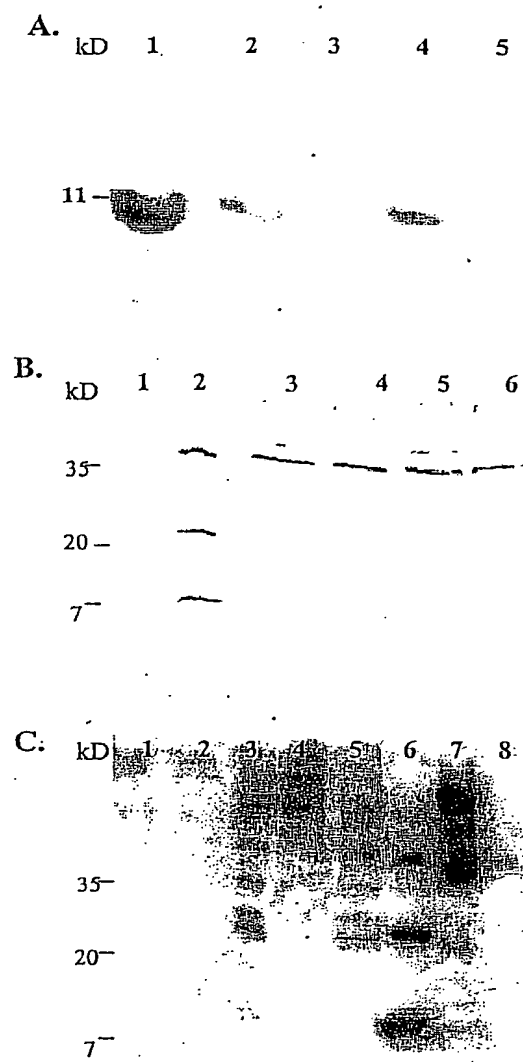
Table 1: Unmodified native codon composition and expression levels observed in transgenic chloroplasts. See section d) for details of AT content, %psbA optimal codons and % of codons that match the cp tRNA pool. TSP: % total soluble protein





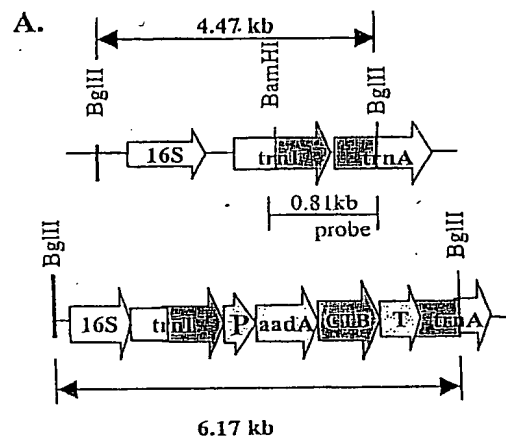
27.

Figure 1

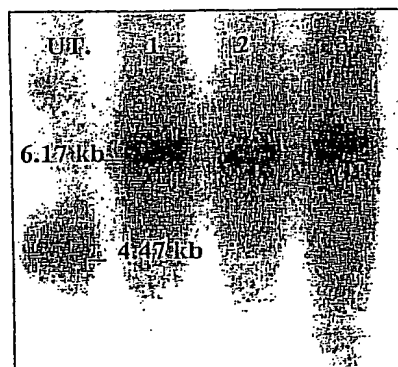


28.

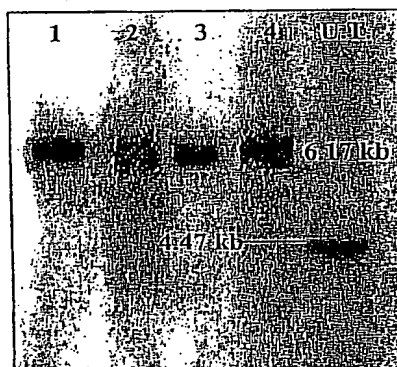
Figure 2



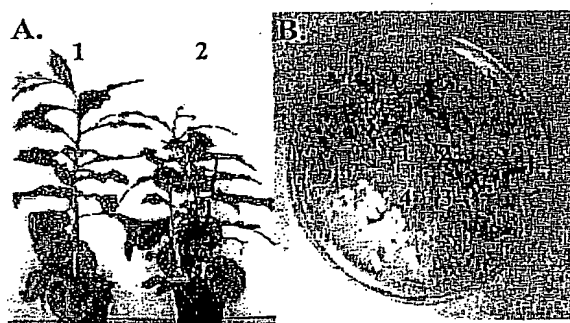
B.



C.

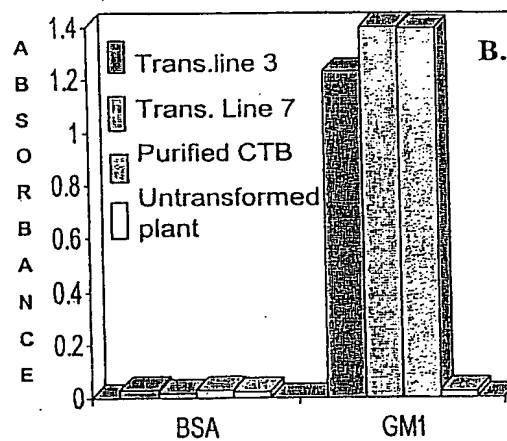
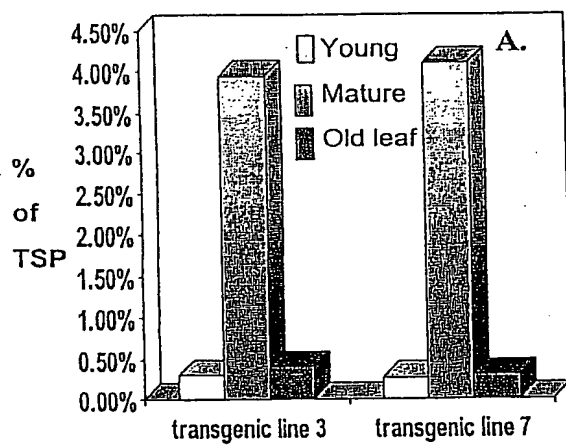


29.  
Figure 3

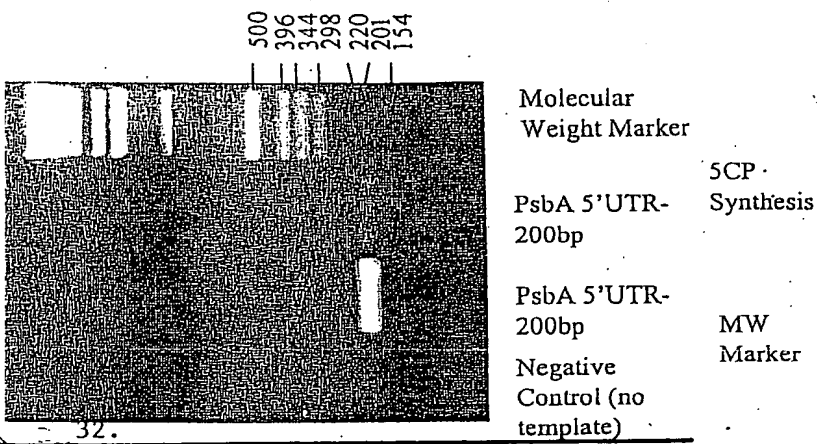


30.  
Figure 4

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31.  
Figure 531



**Figure 1** (above): cloning of the PsbA 5' untranslated (5'UTR) from the chloroplast genome

**Figure 3** (below): a comparison of the DNA sequences of native proinsulin (top) and plastid modified proinsulin (bottom)

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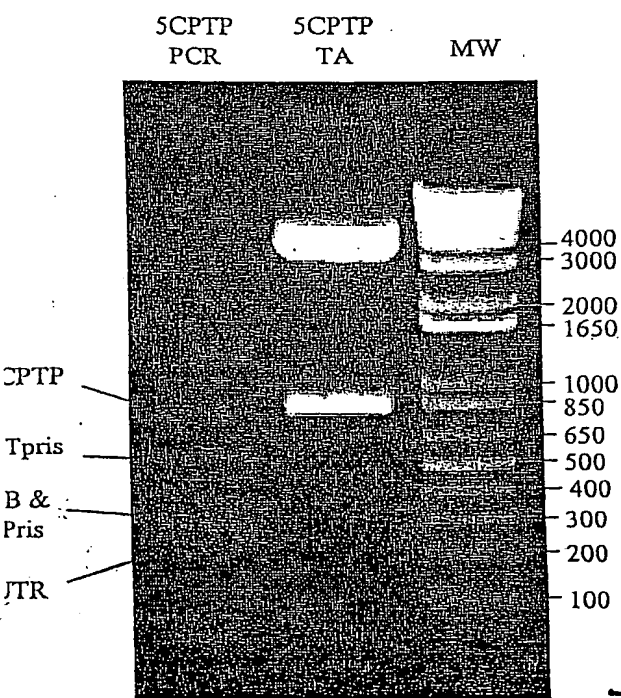
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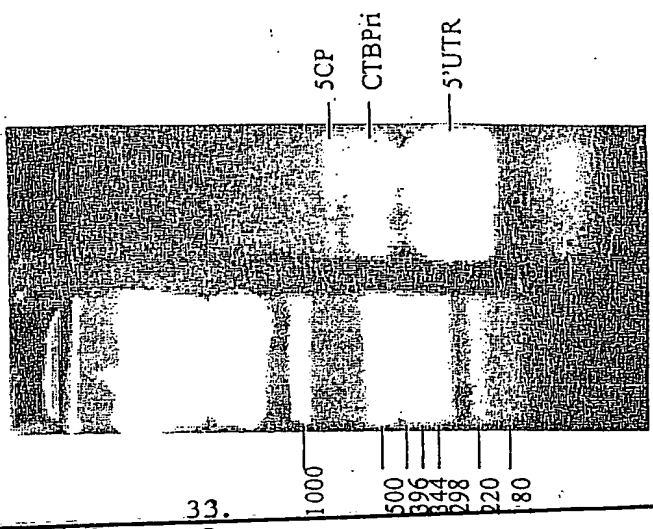
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| | | | | | | | | |
jaaaactactgtaacta   Chloroplast Modified Proinsulin
  
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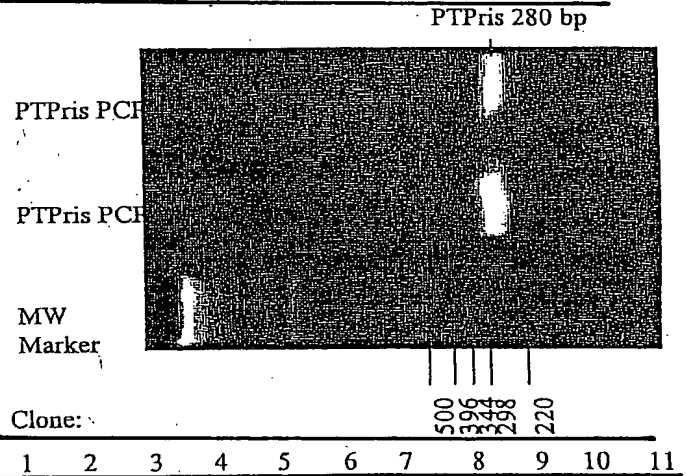
**Figure 6** (above): PCR products to confirm construct integration into the chloroplast genome using two primers, 3P and 3M. 3P anneals to the native chloroplast genome and 3M anneals to the introduced spectinomycin resistance gene, *aadA*, creating a 1600 bp product only in transgenic clones

**Figure 5** (left): SOEing of the 5'UTR, CTB, and plastid modified proinsulin, which results in the fusion of all three sequences denoted as 5CPTP. The second lane show this



**Figure 2** (above): SOEing of the 5'UTR to the CTB- human proinsulin sequence. 5CP is the PSbA 5'UTR and the Cholera Toxin B subunit (CTB) human proinsulin fusion

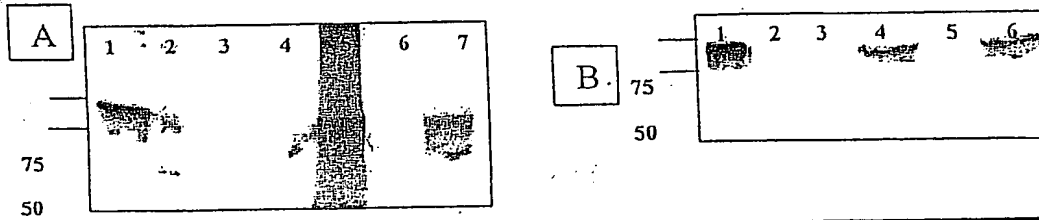
**Figure 4** (below): Recursive PCR to synthesize the chloroplast modified proinsulin (Ptpri)





promoter. IFN $\alpha$ 5 gene was cloned into the pLD using both sequences and bombarded into tobacco leaves. Shoots appeared after 5 weeks and the second round of selection is in progress.

• Expression of HSA via the chloroplast genome in tobacco.



38.

Figure 1: *Western Blot* of tobacco protein extracts. A) 1: 40 ng pure HSA; 2: molecular weight marker; 3,4,6: untransformed plant extracts; 5: extract from plants transformed with: PLD- 5'UTR-HSA; 7: pLD-Orf1Orf2-HSA. B) 1: 40 ng pure HSA; 2: molecular weight marker; 3,5: untransformed plant extracts; 4: extract from plants transformed with: PLD- RBS-HSA; 6: pLD-Orf1Orf2-HSA. 10 micrograms of plant protein were loaded in each well.

# • Southern blot analysis of HSA transgenic tobacco plants.

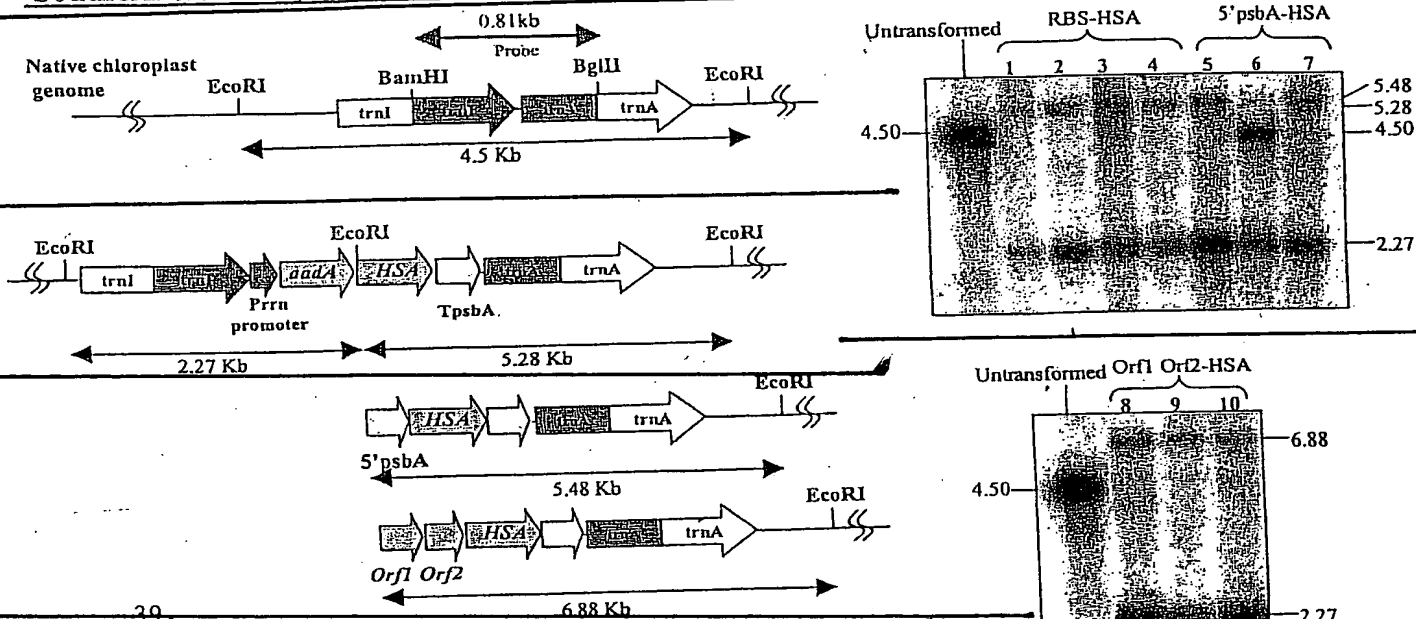


Figure 2: Southern Blot of HSA transgenic plants. Untransformed tobacco DNA vs. transgenic tobacco DNA digested with EcoRI. 1,2,3,4: DNA from plants transformed with pLD-RBS-HSA; 5,6,7: pLD-5'psbA-HSA; 8,9,10: pLD-Orf1-Orf2-HSA. Note homoplasmy in all the clones except number 6.

## • Northern blot analysis of HSA transgenic tobacco plants.

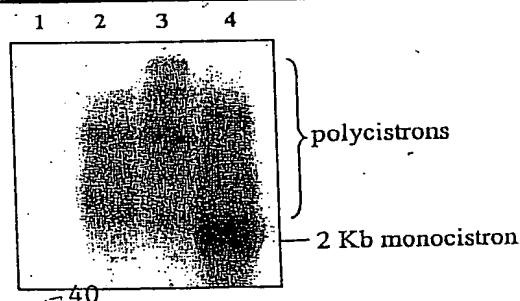


Figure 3: Northern Blot of HSA transgenic plants using HSA probe (1.8 kb). 1: untransformed tobacco RNA. 2: RNA from plants transformed with: pLD-RBS-HSA; 3: pLD-Orf1-Orf2-HSA; 4: pLD-5'psbA-HSA. Note different sizes of transcripts and the presence of monocistrons in number 4.

## • ELISA analysis of HSA transgenic tobacco plants.

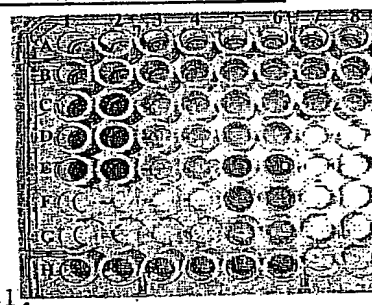


Figure 4: ELISA of HSA transgenic plants. A-E/1-2: HSA standards; F/1-2: Blank; G/1-2: Untransformed Petit Havana protein extracts; D-E/3-4: proteins from plants transformed with pLD-Orf1-Orf2-HSA; F-G/3-4 and D-H/7-8: pLD-RBS-HSA; Rest of the wells contain extracts from different clones transformed with pLD-5'psbA-HSA.

## • IGF-I optimized sequence and PCR product after synthesis of the new gene.

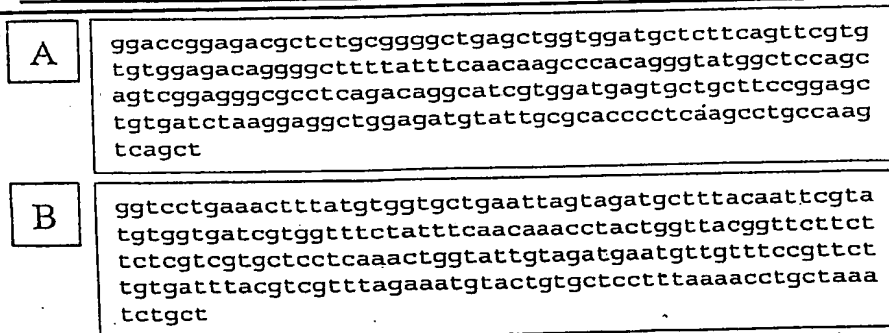


Figure 5: A) IGF-I native sequence coding for the mature protein. B) IGF-I optimized sequence according to chloroplast preferred codon usage. Note changes in red. C) IGF-I synthetic gene after recursive PCR.